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Characterization of the Unique C Terminus of the *Escherichia coli* τ DnaX Protein

MONOMERIC C-τ BINDS α AND DnaB AND CAN PARTIALLY REPLACE τ IN RECONSTITUTED REPLICATION FORKS*

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A contact between the dimeric τ subunit within the DNA polymerase III holoenzyme and the DnaB helicase is required for replication fork propagation at physiologically-relevant rates (Kim, S., Dallmann, H. G., McHenry, C. S., and Marians, K. J. (1996) *Cell* 84, 643–650). In this report, we exploit the OmpT protease to generate C-τ, a protein containing only the unique C-terminal sequences of τ, free of the sequences shared with the alternative γ frameshifting product of *dnaX*. We have established that C-τ is a monomer by sedimentation equilibrium and sedimentation velocity ultracentrifugation. Monomeric C-τ binds the α catalytic subunit of DNA polymerase III with a 1:1 stoichiometry. C-τ also binds DnaB, revealed by a coupled immunoblotting method. C-τ restores the rapid replication rate of inefficient forks reconstituted with only the γ *dnaX* gene product. The acceleration of the DnaB helicase can be observed in the absence of primase, when only leading-strand replication occurs. This indicates that C-τ, bound only to the leading-strand polymerase, can trigger the conformational change necessary for DnaB to assume the fast, physiologically relevant form.

DNA replication in *Escherichia coli* occurs in a rapid, accurate and highly coordinated reaction. The rate of replication fork progression *in vivo* is about 1000 nucleotides per second at 37 °C (1). Replication of the leading strand occurs nearly continuously, whereas the lagging strand is synthesized as a series of RNA-primed Okazaki fragments of about 2000 nucleotides that are subsequently processed and ligated into high molecular weight DNA.

The elongation reaction is catalyzed by the DNA polymerase III holoenzyme, an enzyme that alone on SSB[1]-coated templates can replicate DNA at nearly the rate of fork progression (2). The holoenzyme is composed of three principal subassemblies. The pol III core is composed of the α catalytic subunit, the ε 3′→5′ proofreading exonuclease and θ (3, 4). β is the processivity factor (5); it encircles DNA and contacts the polymerase, tethering it to the template (6). At high concentrations, β alone can assemble with pol III to confer high processivity, but under physiological conditions β requires a multiprotein ATPase, the DnaX complex, for its efficient assembly on primer-termini (7).

The DnaX complex contains a tetramer of the DnaX protein, plus one subunit each of δ, δ′, χ, and ψ (8, 9). The *dna*X gene encodes two proteins γ and τ of 47.4 and 70 kDa, respectively (10, 11). The shorter γ product arises from programmed ribosomal frameshifting (12–15). DnaX complex containing only the γ DnaX protein (γ complex; $\gamma_4\delta\delta'\chi\psi$) can efficiently assemble β onto DNA, but lacks the ability to form a stable contact with the polymerase. γ complex, in the absence of τ, can also remove β_2 prematurely from a complex with replicating DNA polymerase III core, decreasing its processivity (16). Within holoenzyme, the DnaX complex contains both *dna*X gene products in a stoichiometry of $\gamma_2\tau_2\delta\delta'\chi\psi$ (8, 9). The τ protein contacts α tightly, forming a dimeric enzyme that effectively couples the leading- and lagging-strand polymerase (17, 18). The τ protein is also necessary to reconstitute a holoenzyme that behaves asymmetrically in the presence of ATPγS, a property that has been postulated to arise from distinct leading and lagging-strand polymerases within the holoenzyme (19). At reconstituted replication forks on duplex DNA, the τ protein is essential for rapid replication and coupled leading- and lagging-strand synthesis (18, 20). τ is also required for efficient retargeting of the lagging-strand polymerase to the next primer during Okazaki fragment synthesis, permitting the same polymerase to synthesize multiple Okazaki fragments in sequence (18). It has been assumed that this function of τ arose from its ability to bind tightly to α and to dimerize within the natural DnaX complex, linking the two polymerases (8, 17, 21).

An essential contact required for rapid replication fork movement occurs between τ and DnaB, the replication fork helicase. Within replicating DNA polymerase III holoenzyme, the τ-DnaB contact triggers the assumption by DnaB of an alternative fast form conformation that accelerates its rate of duplex DNA unwinding an order of magnitude to near the rate of fork progression *in vivo*. Neither τ alone in the absence of DNA polymerase III nor γ even in the presence of polymerase can accomplish this DnaB acceleration function. Binding studies revealed that τ and DnaB could form a moderately strong complex free in solution by themselves; γ and DnaB could not

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[1] The abbreviations used are: SSB, single-stranded DNA-binding protein; holoenzyme, DNA polymerase III holoenzyme; pol III, DNA polymerase III; TFII, tailed form II DNA; DTT, dithiothreitol; BSA, bovine serum albumin; ATPγS, adenosine 5′-*O*-(3′-thiotriphosphate); NTP, nucleotide triphosphate; PBS, phosphate-buffered saline.

(20). The DnaB-τ contact was also essential to restore proper Okazaki fragment length to the products of *in vitro* reactions (18). This is presumably a direct consequence of the rate effect. DnaB associates with the primase, leading to the synthesis of RNA primers (22, 23). If the frequency of DnaG-DnaB encounters is constant, a more slowly moving helicase will mediate formation of primers that are more closely spaced, resulting in shorter Okazaki fragments.

The DnaB helicase can be assembled onto DNA by two pathways. The DnaA protein can act at the *E. coli* replication origin, *ori*C, on a DnaB-DnaC complex to assemble DnaB onto DNA, establishing two replication forks that progress around the chromosome (24). The PriA, B, C, and DnaT proteins act on a DnaB-DnaC complex in an alternative pathway to assemble the DnaB helicase on DNA and to re-establish stalled replication forks (25). This latter system has also been exploited by ϕX174 as part of its replication machinery (26, 27).

Because τ, but not the shorter γ DnaX protein interacts with DnaB and α, it would be expected that the unique C terminus of τ not found in γ is responsible for these interactions. *E. coli* OmpT protease cleaves τ between two lysines located at the C terminus of γ to generate γp, a protein only two amino acids shorter than authentic γ and C-τ, the unique C-terminal element of τ (28). We exploited OmpT protease to generate sufficient quantities of C-τ for its purification and physical and functional characterization. This permitted an assessment of the contributions of C-τ independent of the γ portion of τ.

EXPERIMENTAL PROCEDURES

Reagents, DNAs, Enzymes, and Replication Proteins—Alkaline phosphatase was from Roche Molecular Biochemicals. Single-stranded circular DNAs from bacteriophages f1AY-7/M and f1R229-A/33 were prepared as described previously (29). PriA, PriB, PriC, DnaT, DnaB, DnaC, and DnaG were purified as described (30). Pol III HE subassemblies and subunits were purified as indicated: core (31), β (32), τ and γ (19), δ and δ′ by an unpublished procedure,[2] and χψ (33). The *E. coli* single-stranded DNA-binding protein (SSB) was purified according to Minden and Marians (34).

Buffers—Buffers were: buffer ompT, 50 mM HEPES-KOH (pH 7.4), 5% glycerol, 50 mM NaCl, 1 mM DTT, 1 mM $Mg(OAc)_2$, 0.01% Nonidet P-40; Buffer A, 50 mM HEPES-KOH (pH 7.4), 5% glycerol, 20 mM NaCl, 1 mM $Mg(OAc)_2$; Buffer HKGM, 50 mM HEPES-KOH (pH 7.4), 100 mM potassium glutamate, 10 mM $Mg(OAc)_2$, 10 mM HEPES-KOH, 150 mM NaCl, 3.4 mM EDTA, 0.005% P-20 detergent (Pharmacia Biosensor); Buffer R, 62.5 mM Hepes-KOH (pH 7.9), 15 mM $Mg(OAc)_2$, 12.5 mM DTT, 125 μg/ml BSA, 105 mM KCl, 6.25 mM ATP, 50 mM dCTP, and 50 mM dGTP.

Preparative Scale Production of C-τ—In order to produce large amounts of C-τ the protocol described in the legend to Fig. 1 was scaled up. τ (15 mg, 2.2 ml, 6.8 mg/ml) was diluted into pre-warmed Buffer ompT (40 ml) in a screw-top centrifuge bottle. OmpT protease (4.5 μl of a 2 mg/ml stock) was added and the solution mixed by gentle inversion. The reaction was incubated at 30 °C for 5 min, quenched by the addition of 20 ml of an ice-cold solution of ammonium sulfate (0.7 g/ml in water) and placed in an ice-water slurry. An additional 10 g of solid ammonium sulfate was added to the solution in order to completely precipitate the protein. After 30 min on ice, the precipitated protein was collected by centrifugation (30 min, 22,000 × *g*). The pellet was flash frozen in liquid nitrogen and stored at −80 °C.

Purification of C-τ—The ammonium sulfate pellets were dissolved in 40 ml of buffer A (conductivity equivalent to 40 mM NaCl) and centrifuged (28,000 × *g*) to clarify. This solution was applied to an SP-Sepharose column (8 × 2 cm), which had been equilibrated in buffer A, at a flow rate of 48 ml/h. The column was washed with 50 ml of buffer A + 100 mM NaCl at a flow rate of 33 ml/h and eluted with a 12-column volume linear gradient of 100 → 250 mM NaCl in buffer A at a flow rate of 25 ml/h; 4-ml fractions were collected. C-τ (4.3 mg) eluted between fractions 42 and 50 (conductivity about 150 mM NaCl). Most of the γp (6.7 mg) produced by the ompT cleavage flowed through the column (fractions 5–10). The remaining γp (3.0 mg) eluted during the wash step (fractions 22–25). Remaining uncleaved τ (1.8 mg) eluted between fractions 54 and 62 (conductivity ~200 mM NaCl).

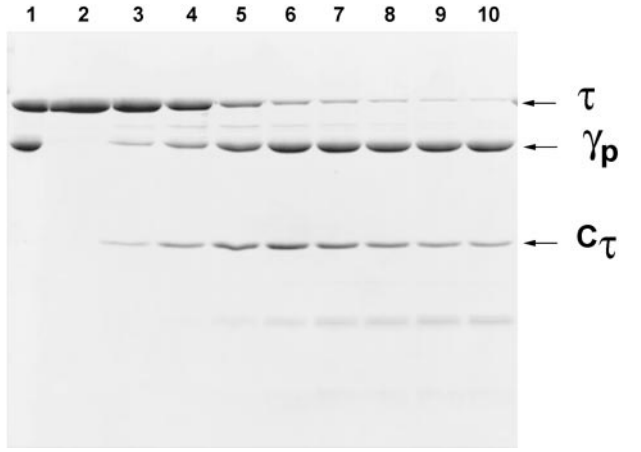

FIG. 1. **Time course for creation of C-τ by digestion of τ with OmpT protease.** *Lane 1*, τ (*upper band*), and γ (*lower band*) markers. Digestion of individual aliquots of τ (6.8 μg) by OmpT protease (4 ng) was conducted in 10 μl of Buffer ompT (total volume). Reactions were allowed to proceed for 0, 1, 2, 5, 10, 15, 20, 25, and 30 min, at 30 °C, then immediately quenched by the addition of 5 μl of SDS sample buffer (*lanes 2–10*, respectively). Samples were boiled and analyzed by electrophoresis through a 12% SDS-polyacrylamide gel.

Fractions 42 to 50 inclusive, containing C-τ, were pooled (32 ml, 2.1 mg) and solid ammonium sulfate (16 g, 0.5 g/ml of solution) was added to precipitate C-τ. The protein was collected by centrifugation (28,000 × *g*), then dissolved in 0.5 ml of buffer A. This solution was clarified by centrifugation (28,000 × *g*), then chromatographed over a Superose 6 HR 10/30 gel filtration column (24 ml) equilibrated with buffer A. The column was developed in buffer A at a flow rate of 12 ml/h: 0.5-ml fractions were collected. Trace quantities of τ and γp contaminating the C-τ fractions from the SP-Sepharose column were removed during Superose 6 gel filtration. C-τ eluted between fractions 33 and 36 (2.0 mg). Each fraction was divided into 25-μl aliquots, flash frozen in liquid nitrogen and stored at −80 °C.

Analytical Ultracentrifugation of C-τ, α, and C-τα complex—Analytical ultracentrifugation was used to characterize the biophysical properties and native oligomerization states of C-τ, α, and the complex between C-τ and α. The Beckman Optima XL-A analytical ultracentrifuge (An-Ti60 rotor) was used for all experiments. Sedimentation velocity and equilibrium experiments on C-τ were performed in buffer A. Sedimentation equilibrium experiments with α and the C-τα complex were performed in buffer HKGM + 5% glycerol and 0.5 mM DTT. All experiments were performed at 4 °C.

Sedimentation velocity analysis of C-τ was performed using 5, 2.5, and 1.25 μM concentrations of C-τ at 40,000 rpm, using double sector cells. Radial absorbance scans were taken at 20-min intervals at 230 nm. Data were analyzed using the second moment/boundary spreading method (35) provided with the Beckman analysis software. The values for $s^0_{20,w}$ and $D^0_{20,w}$ were determined by linear regression of the apparent *s* and *D* values at each C-τ concentration to zero protein concentration and adjustment to the standard conditions (water, 20 °C) (36).

Sedimentation equilibrium experiments were done using six-sector cells. Radial absorbance scans (280 nm for C-τ and 235 nm for α and α·C-τ) were taken for data analysis only when invariant scans taken 6 h apart indicated that equilibrium had been reached (typically >24 h). C-τ, α, and the C-τα complex (prepared by mixing equimolar amounts of α and C-τ) were each centrifuged at three different concentrations and at three different rotor speeds (C-τ: 18,000, 24,000, and 36,000 rpm, 5, 2.5, and 1.25 μM; α: 6000, 8000, and 10,000 rpm, 500, 250, and 125 nM; C-τα complex: 6000, 8000, and 10,000 rpm, 500, 250, and 125 nM). The resulting nine data sets for each protein were analyzed simultaneously by nonlinear least squares curve fitting using the program MLAB (Civilized Software Inc., Bethesda, MD). Data for each protein and the complex could be fit to Equation 1. This model describes a single, ideal, sedimenting species,

$$A(r) = A_o \exp((1 - \nu\rho)(\omega^2/2RT)M(r^2 - r_0^2)) + E \qquad \text{(Eq. 1)}$$

$A(r)$ is absorbance at radial position, r; A_o is absorbance at radial position, r_0; ν, protein partial specific volume; ρ, solvent density; ω = rotor angular velocity; *r* , gas constant; *T,* temperature (K); *M,* native

[2] M. Song and C. McHenry, manuscript in preparation.

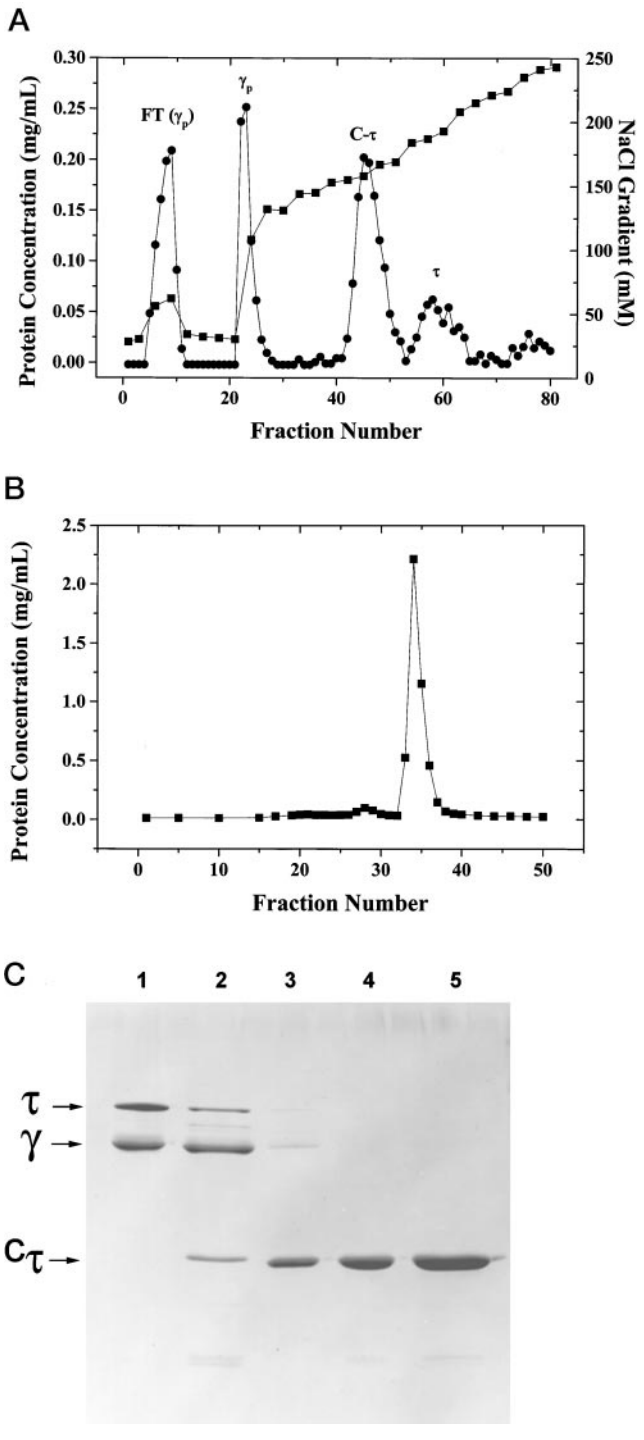

FIG. 2. **Purification of C-τ.** *A*, SP-Sepharose chromatography. As described under "Experimental Procedures," the ammonium sulfate precipitate resulting from digestion of 15 mg of τ was applied to an SP-Sepharose column and resolved with a linear gradient of increasing salt. The plot indicates the salt gradient, the distribution of protein and the identity of protein peaks as determined by SDS-polyacrylamide gel electrophoresis. A portion of γp flowed though the column (*FT*); the remainder eluted in the salt gradient. *B*, Superose 6 gel filtration. C-τ recovered from SP-Sepharose chromatography was subjected to Superose 6 gel filtration to eliminate trace contamination by τ_4. The distribution of protein is shown. *C*, SDS-polyacrylamide gel electrophoresis of fractions obtained during the purification of C-τ. Samples from each step of the C-τ purification were denatured and subjected to electrophoresis on a 13% SDS-polyacrylamide gel. Protein was detected by Coomassie Blue staining as described under "Experimental Procedures." *Lane 1*, τ and γ protein standards (2.5 μg each protein). *Lane* 2, τ digested with ompT protease (5 μg of protein). *Lane 3*, SP-Sepharose pool (3 μg of protein). *Lanes 4* and *5*, Superose 6 pool (3 and 6 μg of protein, respectively).

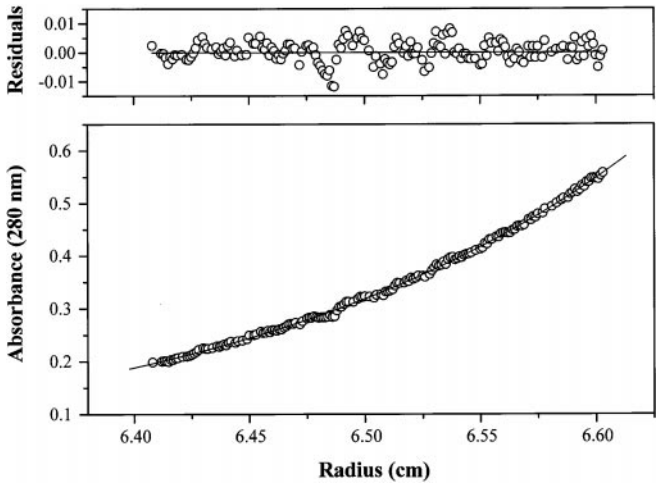

FIG. 3. **Sedimentation equilibrium analytical ultracentrifugation of C-τ.** Concentration distribution of C-τ at sedimentation equilibrium. *Circles* represent the actual data points and the *line* represents the theoretical fit. A summary of the data is shown in Table I. C-τ (5, 2.5, and 1.25 μM) was centrifuged (18,000, 24,000, and 36,000 rpm) and fit to Equation 1 as described under "Experimental Procedures." The example shown is 2.5 μM C-τ sedimented at 18,000 rpm.

molecular weight; and *E,* baseline offset.

Additional models describing self-association were also considered in the analysis of the C-τ sedimentation equilibrium data. The data was fit to models describing monomer-dimer, monomer-trimer, and monomer-tetramer self-association according to Equation 2 (37),

$$A(r) = A_o \exp(\mathrm{BM}(r^2 - r_0^2)) + A_o^n \exp(\mathrm{LNK_A} + n\mathrm{BM}(r^2 - r_0^2)) + E \quad \text{(Eq. 2)}$$

$B = (1\text{-}\nu\rho)(\omega^2/2RT)$; $\mathrm{LNK_A}$ is the natural log of the association constant and n is the oligomeric model under consideration ($n = 2$, monomer-dimer; $n = 3$, monomer-trimer; $n = 4$, monomer-tetramer).

Superose 6 Gel Filtration of C-τ with DnaB Helicase—All gel filtration studies were performed at 4 °C on an Amersham Pharmacia Biotech FPLC system. Standards and protein complexes were chromatographed by gel filtration on a Superose 6 HR 10/30 (24 ml; Amersham Pharmacia Biotech) FPLC column. The column was developed in buffer HKGM at a flow rate of 0.2 ml/min and 0.5-ml fractions were collected from the point of injection. DnaB and C-τ were mixed by gentle vortexing and allowed to incubate at room temperature for 15 min before chromatography as described above.

Rolling Circle DNA Replication Assay—Tailed form II (TFII) DNA was prepared as described by Mok and Marians (38). Reaction mixtures (12 μl) containing 50 mM HEPES (pH 7.9), 12 mM MgOAc, 10 mM DTT, 5 μM ATP, 80 mM KCl, 0.1 mg/ml BSA, 1.1 μM SSB, 0.42 nM TFII DNA, 3.2 nM DnaB, 56 nM DnaC, 680 nM DnaG, 28 nM DnaT, 2.5 nM PriA, 2.5 nM PriB, 2.5 nM PriC, and the pol III HE, HE subunits, or subassemblies at 28 nM unless indicated otherwise, were preincubated at 30 °C for 2 min. NTPs were added to final concentrations of 1 mM ATP, 200 μM GTP, 200 μM CTP, and 200 μM UTP, and dNTPs to 40 μM and the reaction was incubated for 2 min at 30 °C. [α-^{32}P]dATP (2000–4000 cpm/pmol) was added to the reaction mixture and the incubation was continued at 30 °C for an additional 10 min. DNA synthesis was quenched by addition of EDTA to 40 mM. Total DNA synthesis was determined by assaying an aliquot of the reaction mixture for acid insoluble radioactivity. DNA products were analyzed by alkaline gel electrophoresis as described (39).

Isolation of β-Template DNA Complexes—A reaction mixture (120 μl) containing 0.83 nM TFII DNA, 82 nM β, 41 nM γ-complex, 1.1 mM SSB, 50 mM Hepes-KOH (pH 7.9), 12 mM $Mg(OAc)_2$, 10 mM DTT, 2 mM ATP, 80 mM KCl, 0.1 mg/ml BSA, 200 mM each of GTP, CTP, and UTP, and 40 mM dCTP and dGTP (all 4 NTPs and the 2 dNTPs are included just so that the pooled material would be in the same reaction buffer as the rolling circle reaction) was incubated for 10 min at 30 °C and then filtered through a 5-ml Bio-Gel A-150m (Bio-Rad) column equilibrated with Buffer R. Fractions (100 μl) containing excluded material were identified by locating the ^{3}H-TFII DNA and the peak fraction was used for assay.

Replication Fork Rates—An aliquot (110 μl) of the peak fraction containing the β·TFII DNA complex was incubated in a total reaction

TABLE I
Physical properties of C-τ, α, and C-τα complex

Property	Method	C-τ	α	C-τα complex
Subunit molecular weight	Calculated from sequence	23,741	129,900	153,641 g/mol
Quaternary structure	Sedimentation equilibrium[a]	Monomer	Monomer	$(C\text{-}\tau)_1\text{-}(\alpha)_1$
Native molecular weight	Sedimentation equilibrium[a]	20,745 ± 49	114,886 ± 1036	132,687 ± 693[b]
Native molecular weight	Sedimentation velocity[a,c]	26,950 g/mol	ND[d]	ND[d]
Partial specific volume, ν	Calculated from sequence[e]	0.739 $cm^3\ g^{-1}$	0.739 $cm^3\ g^{-1}$	0.739 $cm^3\ g^{-1}$
Sedimentation coefficient, $s^0_{20,w}$	Sedimentation velocity[a,c]	2.93 ± 0.28 S	ND[d]	ND[d]
Diffusion coefficient, $D^0_{20,w}$	Boundary spreading[c]	1.02 ± 0.06 × 10^{-6} $cm^2\ s^{-1}$	ND[d]	ND[d]
Stokes radius	From $D_{20,w}$	21 Å	ND[d]	ND[d]
Frictional ratio, f/f_{min}	From $D_{20,w}$ and M	1.1	ND[d]	ND[d]

[a] Performed as described under "Experimental Procedures."
[b] If the experimentally determined partial specific volumes reported above in Footnote [e] are used to estimate the partial specific volume of the α·C-τ complex (υ = 0.768), a molecular weight of 151,088 ± 789 is obtained. This is probably a more accurate estimate.
[c] Muramatsu and Minton (35).
[d] ND, not determined.
[e] Calculated according to Cohn and Edsall (42). The values obtained for the molecular weight are somewhat lower than the true known monomeric molecular weights, calculated from the sequences of α and C-τ, most likely because of underestimation of the partial specific volume of these proteins. If the monomeric molecular weights are substituted into Equation 1 and the experimental data fit to determine partial specific volumes, values of 0.770 and 0.767 were obtained for C-τ and α, respectively.

volume of 121 μl with the preprimosomal proteins, SSB, and either core or core plus τ at their standard reaction concentrations for 1 min at 30 °C. NTPs, the two remaining dNTPs, and [α-^{32}P]dATP were then added to their standard reaction concentrations to give a final reaction volume of 132 μl and the incubation continued at 30 °C. Aliquots (20 μl) were withdrawn at the indicated times, the reaction terminated by the addition of EDTA to 40 mM, and the DNA products analyzed by electrophoresis through alkaline-agarose gels. The gels were dried and exposed to PhosphorImager screens and x-ray film.

Determination of the Rate of Helicase Unwinding—The 5′-end of the TFII DNA was labeled with ^{32}P using [γ-^{32}P]ATP and polynucleotidyl kinase after the original 5′-phosphate was removed using alkaline phosphatase. The labeled TFII DNA was digested with *Bam*HI DNA, which cleaves only once. The distance in the 5′-3′ direction between the 5′-end of the TFII and the *Bam*HI cleavage site is 3993 nucleotides, however, only 3685 nucleotides of the fragment is in duplex form. Linearized 5′-[^{32}P]TFII DNA (0.42 nM) was incubated at 30 °C in a reaction mixture (150 μl) containing 50 mM Hepes-KOH (pH 7.9), 12 mM MgOAc, 10 mM DTT, 1 mM ATP, 80 mM KCl, 0.1 mg/ml BSA, and SSB and the preprimosomal proteins at their replication reaction concentrations. Aliquots (12 μl) were withdrawn every 30 s and the reaction terminated by the addition of EDTA. DNA products were analyzed by electrophoresis through 1.2% agarose gels at 5 V/cm for 4 h using 50 mM Tris-HCl (pH 7.9), 40 mM NaOAc, and 1 mM EDTA as the electrophoresis buffer. The gel was dried and autoradiographed.

Immunoblots—The indicated amounts of proteins were spotted onto nitrocellulose paper using an S & S Minifold II dot blotting apparatus and dried. The paper was blocked by incubation with 5% nonfat dried milk in PBS for 1 h at room temperature. DnaB (1.6 μg/ml) was incubated with the blot for 15 min in a solution containing 40 mM Hepes-KOH (pH 8), 12 mM MgOAc, 5 mM DTT, 0.1 mg/ml BSA, 4% sucrose, and 1 mM ATP. Glutaraldehyde was then added to a final concentration of 0.1% and the incubation continued for 45 min. The blot was then washed with PBS + 2% milk and 200 mM glycine and then washed 4 times with PBS + 2% milk. Anti-DnaB antisera (1/2000 dilution) in PBS was incubated with the blot overnight at 4 °C. The blot was washed 5 times with PBS + 2% milk and then incubated with goat anti-rabbit IgG coupled to horseradish peroxidase (1/6000 dilution) in PBS. The secondary antibody was washed out and conjugates detected using an enhanced chemilumenescence kit from Amersham Pharmacia Biotech.

Other Methods—Proteins were determined by the method of Bradford (40) using BSA as a standard. SDS-polyacrylamide gel electrophoresis was performed according to Laemmli (41). Staining and destaining of polyacrylamide gels was as described in Dallmann and McHenry (9).

RESULTS

Production and Purification of C-τ

The τ subunit of the DNA polymerase III holoenzyme can be cleaved by the OmpT protease between two exposed adjacent lysines to generate a protein two residues shorter than authentic γ, γp, and the intact C terminus unique to τ, C-τ (28). We exploited this observation to develop a method for generating sufficient quantities of C-τ to study its physical interactions and functional contributions separate from the γ-portion of the τ subunit. A time course of the digestion showed formation of γp and a product corresponding to C-τ. C-τ was susceptible to further time-dependent cleavage to products of 16.4 and 7.2 kDa (Fig. 1).

To provide adequate quantities of C-τ for purification and further analysis, 15 mg of τ was digested and subjected to chromatography on SP-Sepharose to resolve C-τ from other digestion products and undigested τ (Fig. 2*A*). Because it was essential that even trace levels of τ be removed, we subjected the isolated C-τ to gel filtration chromatography on Superose 6 (Fig. 2*B*). The resulting C-τ was free of detectable γp or τ, as determined by SDS-PAGE (Fig. 2*C*). Under the chromatographic conditions used, τ_4 eluted in fractions 24–25 relative to the profile shown in Fig. 2*B* with no detectable τ eluting in later fractions. This procedure yielded 2 mg of C-τ.

Physical Characterization of C-τ

Equilibrium Sedimentation—Since both γ and τ exist as tetramers free in solution, it is likely that the oligomerization domain exists somewhere within γ and not C-τ. However, we required experimental confirmation of that because our experimental strategy included a determination of the functional contributions of C-τ separate from the ability of native τ to dimerize DNA polymerase III. The data for equilibrium sedimentation of 2.5 μM C-τ is shown in Fig. 3. The data fit best to a model (shown by the *solid line*) where only free monomer existed. Self-association models describing monomer-dimer, monomer-trimer, and monomer-tetramer association were also tested for the data shown in Fig. 3. All self-association models showed poor fits and significantly non-random residuals (data not shown) compared with the fit obtained using Equation 1 (ideal monomer). Specifically, the residual plots displayed convex shapes with residuals at least 2-fold greater than with the monomer fit. This indicates that C-τ monomers do not interact under the conditions used for study of its functional contributions reported below. The experimentally determined native molecular weight for C-τ was 20,745 ± 49 g/mol (Table I).

Sedimentation Velocity Analysis—Purified C-τ was also characterized by sedimentation velocity analysis as described under "Experimental Procedures." The experimentally determined native molecular mass for C-τ was 26.95 kDa, consistent with the molecular mass of 23,741 inferred from the amino acid sequence (Table I). The parameters obtained are consistent with a globular, monomeric protein (Table I).

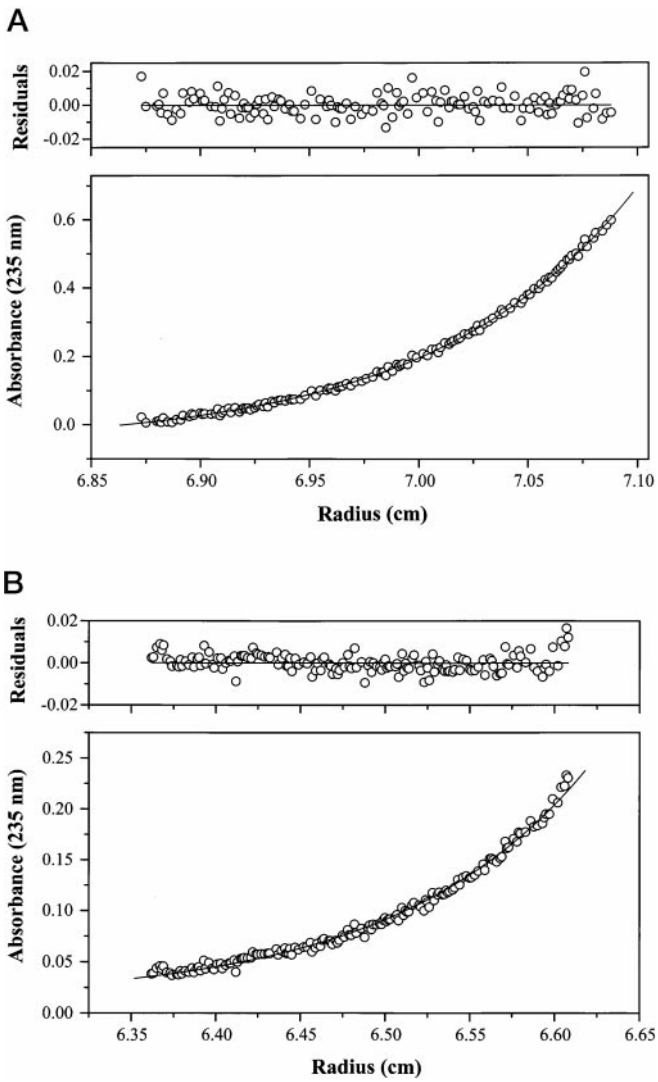

FIG. 4. **Sedimentation equilibrium analytical ultracentrifugation of the C-τα complex.** In all cases, *circles* represent the actual data points and the *line* represents the theoretical fit curve based on Equation 1. A summary of the data is shown in Table I. *A*, concentration distribution of the C-τ·α complex at ultracentrifugal equilibrium. The C-τ·α complex (500, 250, and 125 nM) was centrifuged (6,000, 8,000, and 10,000 rpm) and fit to Equation 1 as described under "Experimental Procedures." The example shown is 500 nM C-τ·α sedimented at 10,000 rpm. *B*, concentration distribution of α at ultracentrifugal equilibrium. The α subunit (500, 250, and 125 nM) was centrifuged (18,000, 24,000, and 36,000 rpm) and fit to Equation 1 as described under "Experimental Procedures." The example shown is 250 nM α sedimented at 10,000 rpm.

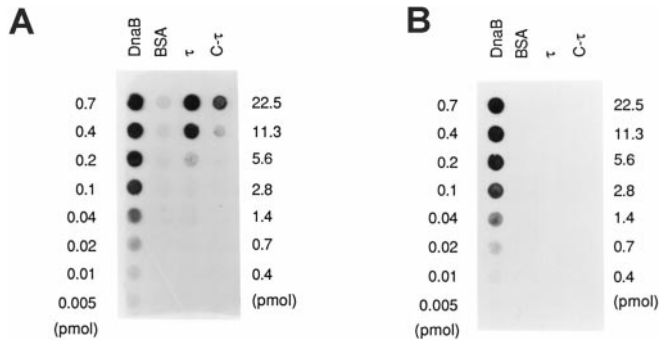

FIG. 5. **Immunoblot analysis of the interaction between τ or C-τ and DnaB.** Immunoblots were performed as described under "Experimental Procedures." *A*, with incubation with DnaB. *B*, without incubation with DnaB. The amounts of protein given on the *left-hand side* of the blots refers to the amount of DnaB applied to the filter. The amounts of protein given on the *right-hand side* of the blots refers to the quantities of all other proteins applied to the filter.

Binding of C-τ to α

Sedimentation Equilibrium Analysis—We prepared complexes of C-τ and α by mixing equimolar quantities of α and C-τ and subjecting them to equilibrium sedimentation analysis (Fig. 4*A*). The data fit best to a model for a single, non-dissociating species (*solid line*) with a molecular weight of 132,687 ± 693, consistent with the sum of the native molecular weights of the α and C-τ proteins determined individually by equilibrium sedimentation (see below).[3] While this is lower than the 153,641 g/mol inferred from the amino acid sequence of α and C-τ, it strongly implies that α and C-τ interact in a 1:1 complex. As a necessary control, we also subjected free α to sedimentation equilibrium. It sedimented as a single non-interacting species of 114,886 ± 1036 g/mol, consistent with the predicted monomeric molecular weight of 129,900 (Fig. 4*B*). The low molecular weight estimates are likely a consequence of the calculated partial specific volumes being low. If the actual monomeric molecular weights were substituted into Equation 1, the experimental data fit to determine the partial specific volumes (see Footnote e of Table I), and these experimentally determined partial specific volumes used for the determination of the molecular mass of the C-τ·α complex, a value of 151 kDa is obtained, very close to the expected 154 kDa calculated value for a 1:1 complex.

Binding of C-τ to DnaB

Gel Filtration Analysis—Upon gel filtration of 16 μM C-τ together with 4 μM $DnaB_6$, we observed both proteins to migrate in the same position as they did alone, indicating no detectable interaction under these conditions (data not shown). Although not a true equilibrium method, these gel filtration experiments indicate a K_d significantly greater than 1 μM for any possible C-τ/DnaB interaction in the absence of additional components. In a previous report (20), we demonstrated a nearly stoichiometric complex between τ_4 and DnaB under the same conditions. We repeated this experiment in parallel as a control with the same results that we initially obtained.

Immunoblot Analysis—The initial observation of a physical τ_4/DnaB interaction was obtained using a procedure where DnaB in solution interacting with immobilized τ was cross-linked with glutaraldehyde and detected by immunological methods (20). We repeated these procedures using C-τ immobilized in parallel with τ_4 and BSA positive and negative controls on a nitrocellulose membrane. Incubation with DnaB, followed by cross-linking and immunodetection using DnaB antiserum indicated an interaction between C-τ and DnaB, albeit weaker than the interaction of DnaB and τ_4 (Fig. 5).

Functional Interactions of C-τ with α and DnaB at Reconstituted Replication Forks

Substitution of C-τ for τ in Reconstituted Replication Forks on Tailed Form II DNA—We have used rolling circle DNA replication to study the action of replication forks reconstituted with purified proteins (20, 22, 38, 39). A tailed form II DNA template is used where a noncomplementary tail of 300 nucleotides carries a primosome assembly site. Thus, when incu-

[3] Alternative models examined included a model where free C-τ and α were in equilibrium and a model where C-τ·α assemblies interacted to form a pol III′-like dimer. The later model can be excluded on the basis of the low average molecular weight obtained. While C-τ and α are certainly in equilibrium with the complex, the dissociation constant is too low to be accessible by this method. Using a BIAcore, we have estimated the K_d to be in the pM range (D. Gao and C. McHenry, manuscript in preparation).

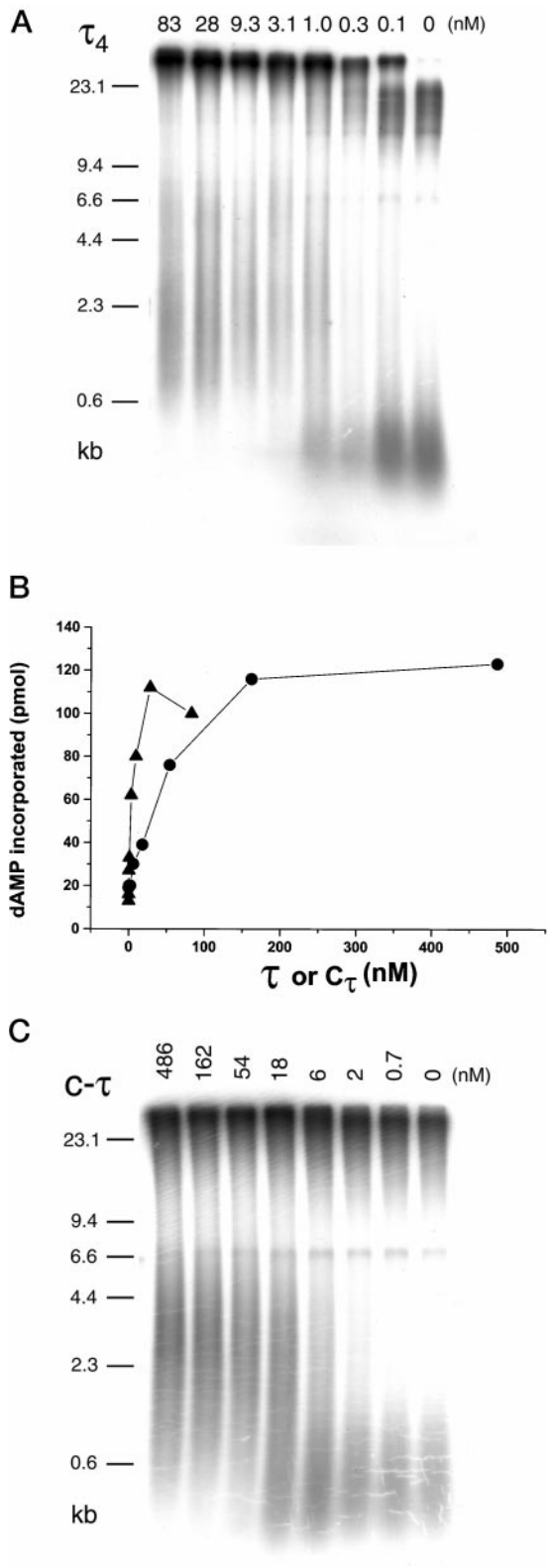

FIG. 6. **The C-τ fragment stimulates rolling-circle replication in the absence of τ and restores Okazaki fragments to normal size.** Standard rolling circle DNA replication reactions were performed as described under "Experimental Procedures" except as noted. *A*, standard rolling circle replication reaction mixtures containing core, β, γ, δ, δ′, χ, and ψ and either no τ (*rightmost lane*) or the indicated concentrations of τ were incubated for 10 min at 30 °C. Reaction products were processed and analyzed by alkaline-agarose gel electrophoresis as described under "Experimental Procedures." *B*, incorporation of [α-^{32}P]dAMP into acid-insoluble product in the reactions shown in *panels A* and *C*. *C*, standard rolling circle replication reactions containing core, β, γ, δ, δ′, χ, and ψ were performed in the absence of τ but in the presence of the indicated concentrations of C-τ. Reaction products were analyzed as described under *A*. The band observed migrating at 6.6 kilobases is unused template that becomes labeled during the reaction.

bated in the presence of SSB, the ϕX174-type primosomal proteins (PriA, PriB, PriC, DnaT, DnaB, DnaC, and DnaG), and the DNA polymerase III holoenzyme, replication generates multi-genome length duplex DNA tails with a long, continuous leading strand and short (about 2 kilobases) Okazaki fragments. These reactions are typically analyzed by electrophoresis through denaturing alkaline-agarose gels where the leading-strand DNA barely enters the gel and the lagging-strand products appear as a distribution centered about 2 kilobases in length (Fig. 6*A*).

τ is required for optimal synthesis at replication forks as revealed by higher rates of dNTP incorporation and longer Okazaki fragments. This can be observed in a positive control reaction where τ is titrated into a reconstituted replication reaction on tailed form II templates (Fig. 6, *A* and *B*). In the absence of τ, DNA synthesis is inefficient and short Okazaki fragments are obtained (Fig. 6*A, rightmost lane*). Because replication forks lacking τ are much slower than those that contain τ (20), the leading strand that is synthesized is also shorter than normal (see the 0.1 and 0 nM lanes in Fig. 6*A*). Increasing concentrations of τ_4 give a breakpoint in a shift to longer Okazaki fragments at about 1–3 nM with an 8-fold stimulation in the rate of dNTP incorporation achieved at saturating concentrations of τ_4 (Fig. 6*B*). When C-τ is substituted for τ_4, a similar pattern emerges (Fig. 6, *B* and *C*), indicating that the monomeric C-terminal domain of τ can substitute for its function in the presence of exogenous γ to assemble β onto the template-primer. As in the case when τ is limiting in the reaction, the slow-moving replication forks formed at low concentrations of C-τ produce shorter leading strands (see the 2, 0.7, and 0 nM lanes in Fig. 6*C*). Because of variability in reaction efficiency, the leading strands produced at limiting concentrations of C-τ were somewhat longer than those produced at limiting concentrations of τ. With C-τ, the breakpoint to longer Okazaki fragment synthesis occurs between 18 and 54 nM with a 6-fold stimulation in overall synthesis occurring at 160 nM C-τ. The apparent K_d of τ_4 and C-τ for replication forks is approximately 3 and 54 nM, respectively, as indicated by the level of 50% maximal stimulation of the synthesis rate (Fig. 6*B*).

Stimulation of DnaB Helicase and Rate of Fork Progression by C-τ—Replication forks were reconstituted in the presence of added C-τ on β-tailed form II molecules that had been separated from free β by gel filtration (16). This ensures that, upon the addition of pol III core and preprimosomal components, only leading-strand DNA replication is obtained. Thus, the rate of replication fork propagation is identical to the change in length of the leading strand as a function of time. Rates were determined from the slope of a plot of the length of the longest leading strand DNA detectable *versus* time over the first 30 s of the incubation (Fig. 7, *A-C*). From this data a rate of fork progression of about 190 nucleotides/s was calculated in the presence of 162 nM C-τ.

τ added alone to DnaB, is ineffective in stimulating its DNA helicase activity (20). For DnaB to be switched to the fast form requires τ to be coupled to replicating DNA polymerase III. Similarly, addition of C-τ to DnaB assembled on tailed form II is not effective in stimulating it to the fast helicase form (Fig. 7*D*). Helicase rates were measured using a tailed form II substrate that had been digested with the *Bam*HI restriction endonuclease. This enzyme cleaves the DNA once, 3685 nucleo-

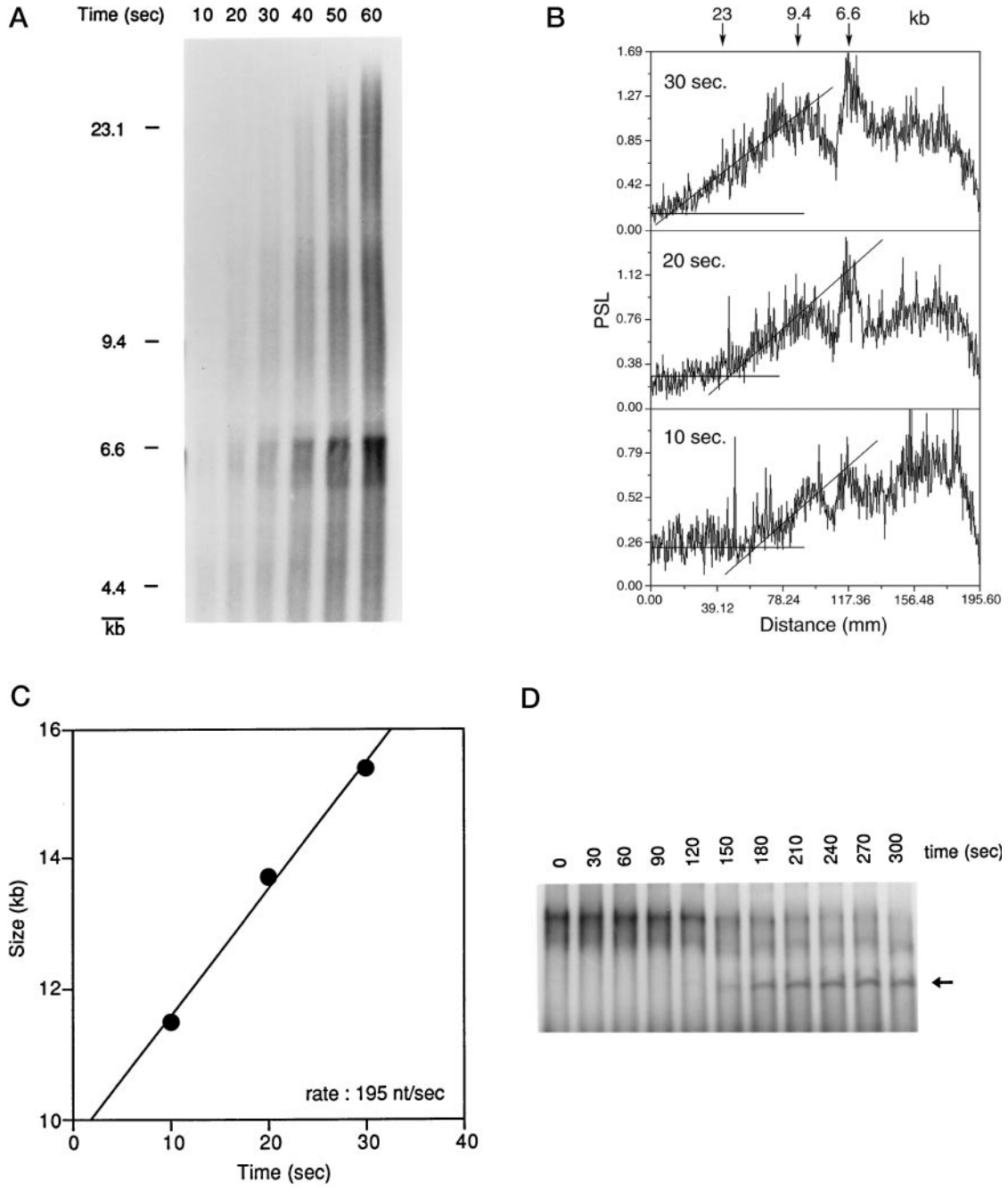

FIG. 7. **C-τ can partially replace τ in increasing the rate of replication fork movement.** β-TFII complexes were incubated in standard rolling circle reaction mixtures containing core, β, γ, δ, δ′, χ, ψ, and C-τ. Aliquots were removed after 10, 20, and 30 s of incubation and the DNA products analyzed by alkaline-agarose gel electrophoresis. Replication fork rate was determined by measuring the size of the longest DNA product present at each time point and plotting this data as a function of time. *A*, denaturing alkaline-agarose gel electrophoretic analysis of the DNA products. *B*, PhosphorImager profiles of the indicated lanes of the gel shown in *panel A*. The size of the longest leading strands present in each lane was determined by locating the *intersection* of a line describing the background level with one describing the slope of the trailing edge of the radioactivity as shown by the intersecting straight lines. *C*, plot of the largest DNA fragments present in the indicated lanes *versus* time to determine replication fork rate. *D*, the C-τ fragment does not affect the rate of preprimosome-catalyzed unwinding of TFII DNA. The rate of preprimosome-catalyzed DNA unwinding in the presence of 200 μM C-τ was determined as described under "Experimental Procedures." The *arrow* indicates the position of the 3685-nucleotide displaced strand.

tides from the start of the duplex portion of the template at the base of the tail. Thus, once a preprimosome is loaded onto the nonhomologous tail, it will unwind the substrate, producing a roughly 3.7-kilobase DNA fragment. The *arrow* in Fig. 7*D* indicates the position of the displaced strand. It first appears at 150 s, indicating an unwinding rate of 25 nucleotides/s in the absence of ongoing replication. This is the same as the rate of DnaB helicase action observed in the absence of τ (20).

Thus, compared with the 190 nucleotide/s rate of fork progression observed, C-τ when coupled to the replication fork mediates a 7-fold stimulation of the DnaB helicase, consistent with the 6-fold increase in radioactive nucleotide incorporation observed in Fig. 6. The experiments reported in Fig. 7 lack primase and ongoing replication on the lagging strand, indicating that monomeric C-τ bound only to the leading-strand polymerase can provide an effective stimulatory signal to the DnaB helicase.

DISCUSSION

We have exploited the ability of the OmpT protease to generate the unique C-terminal domain of τ not found in the alternative *dna*X product, γ. A purification method was devised that yields homogeneous C-τ free of any trace of uncleaved τ. Physical characterization by both equilibrium sedimentation and sedimentation velocity indicated that C-τ is a monomer with no detectable self-interactions and that C-τ forms a 1:1 complex with the α catalytic subunit of pol III. The sedimentation equilibrium data were not consistent with any detectable higher molecular weight forms in equilibrium with the 1:1 complex. This shows that binding of C-τ does not trigger α to dimerize, at least to a detectable level and in the absence of other components found at the replication fork.

Monomeric C-τ was also shown to bind to the DnaB helicase, using an immunoblotting procedure. An interaction of τ with both α and DnaB is essential for it to exert its influence on DnaB helicase rate and proper Okazaki fragment size at the replication fork. C-τ can also bind the same partners and, by that criteria, is properly folded. This permitted us to investigate C-τ's functional contributions separate from τ's ability to dimerize the pol III core and the functional effects of the γ portion of τ. We found that C-τ could replace both τ's ability to restore a normal rate of replication fork progression and its ability to restore Okazaki fragments to their normal length. Approximately 20-fold higher level of C-τ than τ was required. This is presumably a consequence of a weaker apparent DnaB/C-τ interaction. We have observed that more than one C-τ can bind to the DnaB hexamer.[4] Since the apparent K_d of τ_4 for $DnaB_6$ results from the additive energetic contributions of more than one τ subunit interacting with separate DnaB subunits, one would expect the binding of a monomer, even if at undiminished strength, to appear weaker.

The binding of both τ and C-τ to the replication fork apparatus is considerably stronger than the binding of DnaB to C-τ free in solution. Binding of τ to DnaB, detected by gel filtration, although an approximation, appears to be in the 0.1–1 μM range. Weak binding of C-τ to DnaB precludes such an estimate. Nevertheless, the apparent K_d of τ and C-τ for the replication fork, judged by the profiles of their stimulation of replication activity, appears to be 3 and 54 nM, respectively. This indicates a significant (>50-fold) strengthening of the interaction at the fork assembly. This could arise from a different conformation of DnaB and/or τ at the replication fork. Such a strengthening makes biological sense, a tighter affinity of τ for DnaB at the fork would prevent nonproductive associations free in the cytoplasm from competing with the relevant interaction at the fork.

In the absence of primase and lagging-strand synthesis, we have observed that C-τ can stimulate the rate of DnaB progression on forks occupied by only a leading-strand polymerase. This indicates that the τ subunit that contacts the leading-strand polymerase is alone sufficient to provide a signal for triggering the DnaB helicase into the fast mode characteristic of replication forks *in vivo*. However, the diminished affinity of C-τ relative to τ for the replication fork apparatus suggests an additional contact, probably through the τ associated with the lagging-strand polymerase. Whether this putative lagging-strand τ-DnaB contact can also trigger the DnaB helicase into the fast helicase mode or serve as an adjunct in retargeting the lagging-strand polymerase to new primers beyond the established function of dimeric τ in physically coupling the leading- and lagging-strand polymerase is unknown. We also do not know whether C-τ can replace τ in protecting β from premature removal by exogenous γ complex (16). Further experimentation using the valuable new reagent provided by C-τ will be required to resolve these important issues.

[4] D. Gao and C. McHenry, manuscript in preparation.

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